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IMPROVEMENT OF CANOLA MEAL THROUGH REDUCTION OF LIGNIN AND PHYTATE BIOSYNTHESIS

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TECHNICAL AND COMMERCIAL ASSESSMENT OF CANADA - SASKATCHEWAN AGRI-FOOD INNOVATION AGREEMENT PROJECT (APPLICATION NUMBER CODE 96000 403)

Improvement of Canola Meal Through Reduction of Lignin and Phytate Biosynthesis

ANALYSIS OF INTELLECTUAL PROPERTY ISSUES

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The information presented in the present report represents opinion derived as a result of the analysis of publicly available documents and third party information. Although it is believed that the report accurately reflects the current state of the intellectual property related to improvement of canola meal through reduction of lignin and phytate, some information is not available for a variety of reasons. Therefore some of the opinions expressed in this document may not reflect confidential information which may or may not become publicly available in the future. The FAAR Biotechnology Group believes however, that this report accurately reflects the current situation and future prospects but does not warrant that any business, intellectual property or research decision taken by Agriculture and Agri-Food Canada in consideration of this information is justified.

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SUMMARY

The current proposal describes two approaches directed to the improvement of canola seed meal. The reduction of cell wall lignin in the seed coat is contemplated by the transgenic expression of an antisense construct to the enzyme phenylalanine ammonia lyase (PAL). This enzyme is plays a pivotal role in the synthesis of lignin precursors and the reduction of this enzyme specifically in seed coat tissue is expected to effect reduction in lignin accumulation. The enzyme also plays a role in disease resistance and production of substances involved in the response to plant pests.

The second part of the proposal is directed to the reduction of the levels of the anti-nutritional compound phytate. Phytate is the hexaphosphate derivative of *myo*-inositol. Phytate is primarily found in seed and in canola the phytate levels can be relatively high (e.g. up to 4% or more) relative to other meals. The presence of phytate in meal has been linked to a number of detrimental symptoms such as loss of appetite, reduced weight gain and other effects on meal performance . Many of these effects were attributed to the Zn^{+2} binding ability of phytate. The reduction of phytate in canola protein preparations has been difficult by simple genetic means and by processing technologies. Phytate is metabolized poorly by monogastric animals thus reduction of phytate levels in seeds would improve the nutritional quality of canola seed and reduce the polluting effects of phosphate released in animal feces. Phytate also has an anti-nutritional effect by binding cations and may also reduce the ability of animals to fully utilize the protein component of canola meal.

Analysis of existing issued patents and patent applications indicates that the project faces considerable intellectual property barriers and that license to a number of enabling technologies will be required before commercial products can be released.

INTRODUCTION

The current proposal describes two approaches for the improvement of canola meal by reducing lignin and phytate accumulation during seed development. The project will be led by Drs Dan Lefebvre and Stephen Blakely of Performance Plants Inc. and will include collaboration with the Saskatchewan Research Council. The objective of the proposal is to use transformation procedures to introduce recombinant DNA that will function to alter metabolic pathways resulting in the reduction of the levels of lignin and phytate in canola seed.

Although the strategy for the reduction of lignin and phytate both involve the generation of genetically engineered plants and intervention in metabolic pathways the two targets are fundamentally different and trigger the consideration of a number of individual and separate factors. These factors are considered independently however, where appropriate the technologies common to both approaches are considered together.

Alteration of lignin levels Lignin is a complex polymer mainly composed of interconnected units of coniferyl, coumaryl and sinapyl alcohols. The monomers occur in various proportions and with different types of linkages in distinct cells types and in diverse species. There is no single predominant lignin species in plants. Lignins are essential components of plant cell walls where tissues require mechanical strength or are involved in water conduction. Additionally lignins are believed to be involved in mechanisms of resistance to pathogens.

The digestibility of fodder crops and seed meals is influenced by the amount of lignification because lignins are resistant to degradation and act to physically bind the components of the cell wall together. If plant cells walls were considered by analogy to be made of concrete then the lignins would be the steel reinforcing rods. There are many instances in plants where the physical strength of cell walls is not of critical importance thus reduction in lignin content or a change in lignin composition could be beneficial.

Certain types of yellow seeded high oil canolas are known to have a reduced lignin content because the seed coats are in fact thinner. The further reduction of lignin in the seed coat by recombinant means would be an advantage and could result in the elimination of expensive seed hulling steps during seed processing. Generally the use of dehulling techniques places an additional economic burden on processors and is not used in canola meal processing.

However, reduction of lignin alone does not necessarily allow processors to gain value for canola meal. In addition to high lignin content (usually classified as indigestible fibber), there are a number of other anti-nutritional factors that lower the overall value of canola meal. One of the major anti-nutritionals in canola meal is high levels of phytic acid or phytate. This project also proposes to alter phytic acid levels by genetic engineering.

Reduction of phytic acid Phytate (the common name for phytic acid) is the major storage form of phosphorous in canola seed and is produced by the phosphorylation of the sugar alcohol myo-inositol. The biochemistry of phytate formation is not particularly well understood, there are at least two potential pathways for formation and there appears to be a number of different enzyme activities involved in phytic acid biosynthesis. Phytate is found generally in most plant tissues, however is particularly prevalent in seed and pollen. This is expected given its role in phosphorous storage.

Phytate is not removed by standard methods of canola meal production. Phytate is considered an anti-nutrition substance. Monogastric animals such as chickens, pigs and fish do not digest phytate effectively and the phytate may further act to bind important minerals such as zinc. The release of phytate in feces adds to phosphorus pollution in the environment and is a major source of phosphorous runoff. Most microbes can efficiently hydrolize phosphorous from the myo-inositol backbone of phytate thus the phosphorous in phytate is readily released once outside of the animal gut. There is considerable interest in elimination or reduction of phytate using microbial phytase enzymes, either added to meal or produced by recombinant methods. Other new methods of reducing phytate accumulation would be of significant commercial interest.

Phytic acid is associated with a variety of anti-nutritional effects and high phytic acid levels in canola meal preclude its use for various applications and further restrict its inclusion rate for general meal diets. Breeding efforts have been largely unsuccessful to reduce phytic acid, not only in canola meal but most other crops as well. Recently strategies to reduce the antinutritional effects of phytate in meal have been advanced by Mogen through the addition of a microbial phytase enzyme to seed such that the enzyme works in the animal gut, however this approach is not widely applicable to all applications as the content of phytic acid of canola meal remains high. However, this application has shown promise for some applications and will be commercialized within the next two years. A relatively broad US patent issued to Mogen controls the use of microbial phytase enzymes in plant seeds or meal products.

The present application proposes to use a modified kinase (a protein that adds phosphate to various substrates) to alter phytate levels in canola meal. Although this approach has not been tried by other workers, the actual details of how this will be accomplished or what the scientific rationale comprises are not provided in the proposal. Accordingly it is difficult to evaluate the potential opportunities or intellectual property roadblocks for this portion of the project. In general the alteration of plant kinases is not a well-developed art, thus there are no patents that recite alteration of kinases in plant seeds. The particular kinase in question is stated to be a regulatory protein and it is this regulatory function that will be modified as part of this project. The means or rationale for carrying out the particular modifications are not detailed.

TECHNICAL CONSIDERATIONS

The primary applicants Drs Lefebvre and Blakely are experienced plant physiologists and molecular biologists. The research experimentation described for lignin reduction should not present undue difficulties for experienced molecular technicians or postdoctoral fellows working under the guidance of the principal applicants. Experimental results achieved to date by the applicants and by other research groups suggests that the chosen anti-sense approach to reduction in lignin formation has a high rate of success.

The biosynthesis of lignin is part of the phenylpropanoid biosynthetic pathway which is also responsible for production of a wide range of other important compounds including flavonoid pigments, isoflavonoids, phytoalexins and cell division promoting glucosides. The enzyme Phenylalanine Ammonia Lyase (PAL) is involved very early in the pathway and is responsible for the deamination of the amino acid phenylalanine to produce cinnamic acid. This acid is then transformed by hydroxylation and methylation reactions producing different ring substitutions. The resulting phenolics are precursors of the various alcohols that are polymerized into lignin. The biochemistry of the formation of lignins is complex and in addition to PAL a number of other enzymes such as: caffeic acid/5-hydroxyferulic acid-o-methyltransferase (COMT), caffeoyl-CoA-reductase (CCoAOMT), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA-reductase (CCR), peroxidase, 4-coumarate:CoA ligase (4CL) and coniferin-specific beta-glucuronidase (CBG) are currently under study as targets for lignin reduction via antisense suppression in various laboratories.

The possibility that reduction of lignin or changes in lignin composition can be achieved by anti-sense inhibition of an enzyme in the biosynthetic pathway is currently the subject of research at numerous laboratories around the world and elsewhere in Canada. Much of this work is focused on alteration of the lignin content of trees and increases in the digestibility of forages. Research results that have been presented to date and information provided by the applicants provides encouragement that this approach can be commercially useful. As PAL is an enzyme involved in the formation of compounds other than lignin the restriction of the anti-sense to specific tissues, e.g. the seed coat, is a necessary precaution to avoid deleterious effects in the whole plant. The use of a seed coat promoter from a closely related species provides a very good likelihood that appropriate expression can be achieved.

In *Brassica* lignified thickenings occur predominantly in the inner epidermis of the outer integument of the seed coat. In order for the anti-sense approach to PAL to be most effective the chosen promoter should be highly active in these specific cells. Additionally it is required that the biosynthesis of lignin precursors also takes place in the cell coat cells and are not imported from the developing embryo. Preliminary experimental findings suggest that anti-sense to PAL has reduced the content of lignin in tobacco seed coat cells thus the project as described appears promising and should produce positive results.

The applicants correctly identify phytate as an important target for improvement of canola seed meal for feeding monogastric animals. Phytate is typically deposited as discrete globular inclusions in storage microbodies called protein bodies. Although considerable progress has been made in the study of the breakdown of phytate by phytase enzymes relatively little is known concerning the generation of phytate in plants. The applicants describe the modification of a regulatory protein kinase discovered in the laboratory of Dr. Lefebvre by mutagenesis. No detailed information is provided, however, concerning how these changes are expected to affect phytate accumulation. Without this information it is not possible to comment specifically about the possibility of achieving alterations of phytate levels.

The applicants suggest that there will be a number of promising lines produced as a result of the introduction of an altered regulatory kinase. Although much of the basic biochemistry of protein kinases and the regulation of protein cascades by phosphorylation is known in a number of model systems, the involvement of this particular protein kinase in the production of phytate or the regulation of phytate biosynthesis was not presented in the proposal nor was it apparent from the publication lists of the applicants. Accordingly this new protein kinase appears potentially patentable, but the utility and mode of action were not detailed in the application.

INTELLECTUAL PROPERTY SPECIFICALLY RELATED TO LIGNIN REDUCTION

As described above the possibility of reducing the lignin content of plants by down regulation of enzymes involved in biosynthesis has been widely appreciated. The lignin biosynthetic pathway is complicated and a series of different enzymes are being investigated as targets for antisense or other methods of gene suppression.

To date comparatively few patents have issued that relate specifically to lignin reduction, however a number of additional applications have been published. In consideration of the extent of research in this field still further applications that have been filed but not yet published are anticipated.

A US patent, **(5,451,514)** entitled " *Modification of Lignin Synthesis in Plants*" was issued Sept 19, 1995. The inventors are listed as Alain Boudet, Dirk Inze and Wolfgang Schuch and the patent is assigned to Zeneca Ltd of England. The patent describes the control of lignin synthesis by plants using a recombinant antisense gene to an enzyme critical to the synthesis of a lignin precursor. The patent also recites the inhibition by use of a partial sense gene sequence and the increase in lignification by the inclusion of an additional sense gene involved in lignin production.

This patent speaks predominantly to the improvement of the digestibility of fodder crops through lignin reduction but also cites the potential importance of changes in lignin content and composition for making paper from wood and the use of lignins as precursors in chemical synthesis. Although this patent is directed to the improvement of digestibility of plants for animal feed no specific reference is made to alterations of seed coats or improvement of seed meals by reduction of lignin. Reference to canola is made only in the context of possibly increasing grain yield by reducing lignification in general.

Although this patent describes the concept of blocking any enzyme required for lignin biosynthesis the specific embodiments and examples are restricted to the enzyme cinnamyl alcohol dehydrogenase (CAD). The CAD enzyme can catalyze the formation of all the cinnamyl alcohols found in lignin and lignin monomers cannot be synthesized in plants by any pathway not involving CAD. In principle and by the use of inhibitors the CAD enzyme appears to be a good target for lignin reduction, however only data that shows a change in lignin composition is presented in the examples. All of the examples relate to cloning CAD genes and transformation studies in tobacco and as such the broadest claims that have been allowed are restricted to the use of CAD gene sequences for alteration of lignification.

Claim 1 recites blocking the activity of CAD:

1. *A recombinant DNA comprising a gene promoter sequence, and a gene terminator, and an interposed region comprising a nucleotide sequence encoding an mRNA which has sufficient sequence similarity to an endogenous plant cinnamyl alcohol dehydrogenase gene that when said nucleotide sequence is expressed the expression of*

said endogenous plant gene is inhibited, said endogenous plant gene being a gene which encodes cinnamyl alcohol dehydrogenase, and wherein said nucleotide sequence is a plant cinnamyl alcohol dehydrogenase gene or fragment thereof.

Claim 6 is similar but recites the method of lignin reduction.

6. *A method of altering the content or composition of lignin in a plant, comprising stably incorporating into the genome of the plant by transformation a recombinant DNA comprising a gene promoter sequence and a gene terminator, and an interposed region comprising a nucleotide sequence encoding an mRNA which has sufficient sequence similarity to an endogenous plant cinnamyl alcohol dehydrogenase gene such that when said nucleotide sequence is expressed the expression of said endogenous plant cinnamyl alcohol dehydrogenase gene is inhibited, and wherein said nucleotide sequence is a plant cinnamyl alcohol dehydrogenase gene or fragment thereof.*

Other claims are directed to CAD enzymes from tobacco, corn, poplar and eucalyptus and further expand the method to include regeneration of plants from cultured cells. A copy of the patent is appended.

The Zeneca organization has also recently obtained a related patent. US patent **5,633,439** entitled *Expression of Genes in Transgenic Plants Using a Cinnamyl Alcohol Dehydrogenase Gene Promoter* authored by M. Walter was issued May 27, 1997. This patent is directed to the use of the CAD promoter to regulate the expression of recombinant genes in plants. This promoter is considered to have utility for high level of expression of genes in cells and tissues that are subject to lignification. The promoter has also been shown to be inducible by wounding and a number of external stimuli and thus could be useful for the controlled induction of specific gene activities related to pest and disease resistance. The claims are restricted to the use of this specific promoter and as such are not directly related to the current project. A copy of the patent is appended.

The applicants suggest in their proposal that Zeneca has obtained a patent that covers antisense to PAL and that they are pursuing a license agreement. It is not clear what patent they intend to license as the claims of issued US 5,451,514 do not extend to antisense to PAL and thus a license would be unnecessary for the current project. It is possible that a CiP could be issued that will extend the claims further. In this regard a copy of the Canadian application which is the same as the published WPO application was obtained.

Canadian application **2,005,597** entitled Plants Having Reduced Lignin or Lignin of Altered Quality by Schuch et al., assigned to Zeneca is appended. This application is equivalent to the issued US patent but is more general and refers to enzymes other than CAD involved in lignin biosynthesis. In addition the application describes the use of the promoter from the PAL gene (e.g. GPAL2 or GPAL3) to drive the expression of antisense constructs. A citation of Cramer et al., 1989, Plant Molecular Biology 12:376-383 and Bevan et al, 1989, The EMBO Journal 8, no 7., 1899-1906 is given which

suggests that these promoters are in the public domain. (also no patents to the PAL gene itself were recovered in database searches).

In this application the only examples describe antisense to CAD, however, the broadest claim extends to all lignin synthesis enzymes. Claim 1 of the Canadian application recites:

1. A recombinant DNA comprising a plant DNA having a promoter sequence and an insert, located 3' to the promoter and in appropriate reading frame, comprising a nucleotide sequence encoding a mRNA which is substantially homologous or complimentary to mRNA encoded by an endogenous plant gen or a part thereof which encodes an enzyme essential to lignin biosynthesis, so that mRNA transcribed from the said insert inhibits production of the enzyme from the endogenous gene.

If a patent were to issue with such a broad claim then a license from Zeneca would be required.

In addition to the patents and applications directed primarily to CAD , ICI (at that time the parent organization to Zeneca), filed a similar patent application (**WO 93/05160**) entitled Modification of Lignin Synthesis in Plants. This application was authored by Jan Van Doorsselaere et al and is directed to the inhibition of lignin formation by down regulation of the enzyme O-methyl transferase. This application is similar to those described above, the broadest claim, claim 11, recites:

11. *A method for the regulation of lignin biosynthesis in a plant comprising stably incorporating into the genome of a plant by transformation a recombinant DNA encoding the enzyme caffeic acid O-methyl-transferase in sense or antisense orientation under control of a transcriptional initiation region operative in plants.*

A copy of this patent application is appended.

The final patent application that was identified that could have a significant bearing on the current project is **WO 94/08036** entitled *Methods for the Modulation of Lignification Levels*. This application authored by M. Clausen, C. J. Lamb, P. Doerner, Y. El-Kind and R. Megnet is assigned to The Salk Institute for Biological Studies and The University of Oldenberg. This application describes the modulation of plant lignification by regulation of PAL itself and the reduction of lignin precursors by converting them to styrene(s) through the actions of a recombinant phenylalanine decarboxylase (PAD) enzyme. The specific embodiments describe the utility of lignin reduced or increased plants but no specific mention is made to the possible utility of lowering lignin in seed coats.

The PAL gene sequence used was amplified from tobacco using sequence information in the published literature. Expression of antisense PAL genes in tobacco resulted in phenotypic abnormalities in flowers in some of the transformants (6 of 23) and

leaves and flowers (2 of 23). Plants with greatest degrees of abnormal growth contained markedly less lignin. A copy of this application is appended.

The broadest claim recites control of lignification with antisense to PAL.

1. A method of reducing plant lignification, said method comprising introducing into said plant antisense RNA complimentary to PAL-encoding RNA, thereby altering the level of endogenous PAL enzyme in said plant.

If a patent issued with such a claim then a license for the current project would be required.

INTELLECTUAL PROPERTY RELATED TO SEED SPECIFIC EXPRESSION

In order to avoid any possible deleterious effects on the whole plant of repression of PAL activity, the antisense PAL gene is to be restricted in expression to the seed coat. Examination of existing intellectual property related to seed specific expression in *Brassica* was undertaken to determine if any of these patents would impact the present study.

The applicants state that a seed coat promoter from *Arabidopsis* is available but do not state where it is available from or what the terms and conditions may be for using this promoter. It is assumed that the applicants have either characterized this promoter or have obtained this promoter from associates.

The Calgene company has been very active in developing technology for use and modification of canola seed. Three US patents have been issued to Calgene recently that relate to promoters that provide seed specific expression , including those that are active or specific to the seed coat.

The first patent, **US 5,420,034** by J. C. Kridl and V. C. Knauf, entitled Seed-Specific Transcriptional Regulation was issued May 30, 1995. This patent provides for seed-specific gene regulation in order to modulate or modify expression in seed, particularly embryo. The specific embodiments describe the modification of seeds for a variety of commercial purposes from altered oil composition to expression of foreign proteins of interest but no mention is made specifically of changes in the seed coat to improve the nutritional quality of meal. Three types of promoters are derived from Brassicas including the storage protein napin, the acyl carrier protein (ACP) and a seed coat specific promoter EA9. Claim 1 of this patent reads:

1. *A DNA construct comprising in the 5' to 3' direction of transcription, a transcription initiation region from a gene which encodes a product preferentially expressed in a plant seed cell as compared with other plant cells, a DNA sequence of interest other than the native coding sequence of said gene, and a transcription termination region, wherein said gene is a napin gene, an acyl carrier protein gene or an EA9 gene.*

A copy of this patent is appended. The restriction of the issued claims to the exemplified promoters suggests that this patent would not have a direct bearing on the current proposal unless the proposed *Arabidopsis* promoter has sequence homology to EA9.

A second seed specific expression patent has been issued to Calgene that describes the use of another *Brassica* gene identified as Bce4. US 5,530,194 by V. C. Knauf, J. C. Kridl and D. E. Scherer, entitled Sequences Preferentially Expressed in Early Seed Development and Methods Related Thereto was issued June 25, 1996. The patent describes the identification and use of a gene originally isolated from embryo tissue *of B. rapa* that has high levels of expression in the early stages of embryo development. This

particular gene is expressed in embryo and in seed coat. As above this patent would have no bearing on the present proposal unless the proposed *Arabidopsis* promoter and the Bce4 promoter are homologous.

The final US patent, US 5,608,152, by Kridl and Knauf, issued Mar 4, 1997, entitled Seed-Specific Transcriptional regulation is a continuation in part of US 5,420,034. The claims of this patent specifically recite *Brassica* seed but are restricted to the exemplified promoters as described above.

In Europe Calgene has obtained an issued patent (EPO 255 378 B1) by the same authors that has a broader general claim that recites

A DNA construct comprising a seed specific transcriptional initiation region which is from other than the bean phaseolin promoter; a DNA sequence of interest other than the natural coding sequence and a transcriptional termination region.

This claim is sufficiently broad that a license to express a seed coat specific antisense construct would be required in Europe. However, apparently the enforceability of the patent in Europe has been questioned by the European patent office. It is unlikely that this patent will be enforceable.

Overall, it appears that the use of a seed coat promoter will escape any intellectual property associated with seed promoters, unless the exemplifies promoter shows homology with the EA9 or Bce4 promoter covered by the issued Calgene patents.

The phytate portion of the project also relies on a seed-specific promoter and is subject to the same concerns detailed above. It is stated that the mutated regulatory kinase will be placed under the control of a seed specific "promoter from arabin". The derivation of this promoter is not clear. The use of this "arabin promoter", or the source and composition thereof, was not detailed in the proposal. It is not known from the information in the presented if this promoter represents the *Arabidopsis* homologue of the *Brassica* seed gene napin (or cruciferin), or if this promoter is a newly discovered promoter. Recently a patent has been issued to PGS, (US 5623067) which encompasses the *Arabidopsis* 2S albumen, the equivalent of napin. We presume the "arabin" promoter and the promoters claimed in US 5623067 are distinct.

ENABLING TECHNOLOGY REQUIRED FOR USE OF BRASSICA ANTISENSE GENES

Any recombinant DNA approach to *Brassica* seed modification by down regulation of gene activities must involve a number of fundamental parameters that are now very widely used and well entrenched procedures. These techniques are well known to the current applicants and to other biotechnologists, however as they are germane to the current application they are described briefly below.

1) Use of recombinant DNA
2) Individual components of the recombinant DNA and the methods employed for obtaining the genes or DNA fragments in question
3) Methods used to achieve transformation
4) Methods of down regulation of gene expression

1) Recombinant DNA.

The use of recombinant DNA itself, regardless of how achieved, is subject to the Cohen-Boyer patents owned by Stanford University. There is little option for commercial ventures to avoid this restriction, and therefore it is assumed that the applicants have such a license or will obtain one in due course. The patents involved are: **US 4,237,224, 1980, US 4,468,464, 1984, US 4,740,470, 1988**. A license for these patents is required to sell recombinant products into the United States. Stanford has a standard agreement for access to the technology that is available under reasonable terms and conditions.

2) Composition of the Recombinant DNA.

The individual genes and DNA sequences that comprise commonly used components of recombinant DNA molecules are frequently subject to intellectual property rights even though they are widely used and available within the research community. The applicants do not specify the detailed composition of vectors that will be used to deliver the antisense genes but it is probable that some components will be subject to existing patents. Items that require consideration include: border fragments, genes that confer tolerance to antibiotics such as kanamycin or hygromycin or herbicides such as glyphosate or glufosinate, genes that are used as visible markers, e.g. GUS or beta glucuronidase and promoters such as the ubiquitous CaMV 35S. The applicants are aware of these restrictions and it is assumed they would either arrange for commercial use of these genes or find alternatives not subject to rights held by others.

3) Transformation Methods.

The applicants indicate that transformants will be generated by conventional methods which is assumed to mean that they will be generated by co-cultivation of plant explants with *Agrobacteria* carrying binary vectors comprising the antisense constructs of interest.

The use of the binary vector method of *Agrobacterium* mediated transformation of all dicot species is subject to issued US patents of Schilperoort *et al* (US 4,940,838 and US 5,464, 763 transformation assigned to Mogen (now owned by Zeneca). Although there will not be a Canadian patent equivalent (it was not applied for) a license for the use of this method will be required to sell commercial products resulting from this research into the USA and Europe.

A wide variety of other methods have been developed that could be considered for *Brassica* transformation but only the use of microprojectile bombardment could be considered a conventional approach. The use of the 'particle gun technology' originally developed at Cornell and Agracetus is now controlled by E.I. DuPont de Nemours & Co. and a license to practice this method is also required.

In addition the production of transformed *Brassica* plants by any method is subject to the now infamous Moloney transformation patents (e.g. US 5,463,174). At the present time there does not appear to be a way to avoid obtaining a license from Calgene.

4) Down Regulation of Gene Expression

The present project is predicated on the use of antisense RNA as the method of reducing or eliminating the expression of the PAL enzyme. The control of antisense RNA technology in plants, (e.g. US 4,801,540, **US 5,107,065**, US 5,453,566) rests with Calgene and a license to use antisense technology will be required.

It is possible that a license from ENZO is also required as **US 5272065**, Regulation of gene expression by employing translational inhibition of mRNA utilizing interfering complementary mRNA, issued December 21, 1993, by Inouye et al, now controlled by ENZO Biochemicals also claims control of antisense RNA. The Calgene and Inouye patents were the subject of an interference proceeding and the particular application of antisense RNA contemplated in this proposal may be controlled by one or both of the parties. In any event a license will be required to practice antisense RNA. Abstracts and claims to both the Calgene and the Inouye patent are appended.

The equivalent result to antisense may be achieved by use of the Transwitch Technology (US 5,034,323, US 5,283,184) developed by DNAP, however the use of this technology also requires a substantial license agreement.

INTELLECTUAL PROPERTY RELATED TO REDUCTION OF PHYTATE IN CANOLA SEEDS

The second component of the current project is the metabolic intervention in the accumulation of phytate in canola seeds. The reduction of phytate in canola could have considerable benefits as described previously. To date the predominant methods that have been contemplated for reduction of phytate have been largely directed to removal of phytate by the action of phytase enzymes.

Phytase activity is commonly encountered in microorganisms such molds (*Aspergillis*), bacteria (*Bacillus, Pseudomonas*) and yeast (*Saccharomyces*). The removal of phytate by treating plant materials with enzymes mixtures containing microbial phytase is described for example in issued US patent 5,554,399 by E. M. M. Vanderbeke *et al*., entitled Process for Hydrolyzing phytate with a Synergistic Enzyme Composition. A copy of the patent is appended.

The alternative approach of transforming plants to produce microbial phytase has also been accorded patent protection recently. **US 5,593,963** by A. J. J. Van Ooijen *et al* entitled Expression of Phytase in Plants assigned to Mogen International and Gist-brocades was issued Jan 14, 1997. The invention provides for the expression of phytase in transgenic plants. The regulatory sequences include those capable of directing expression either constitutively or in a stage or tissue specific manner. Claim 1 that provides broad coverage recites:

1. *A recombinant expression system capable, when contained in a higher plant cells or the cells of an intact plant, of expressing a first nucleotide sequence encoding a microbial protein which catalyzes the liberation of inorganic phosphate from myo-inositol hexa kis-phosphate, said expression system comprising said first nucleotide sequence encoding said protein operably linked to transcription controlling nucleotide sequences operable in a higher plant cell or in cells of a higher plant.*

A copy of this patent is appended.

In the present application the alternative approach of modifying phosphate metabolism by mutating a regulatory protein kinase is suggested. Mutations in substrate binding sites are expected to produce enzymes that are always on or always off. these mutated enzymes are anticipated to be introduced into transgenic plants under the control of the seed specific arabin promoter. Transformed lines will then be tested for altered phytate levels. Unfortunately insufficient information is provided in the application to appreciate how these modified enzymes will effect the changes in phytate levels. It is assumed that the process would divert or limit the availability of phosphate for the phytate biosynthetic system.

Without additional information it is not possible to properly assess the intellectual property that may govern this specific approach, however, all of the enabling technologies

that relate to the production of transformed *Brassica* plants cited above would also apply here.

A search of the World and US patent databases only revealed three patents that are related to plant protein kinases for the purposes of altering a plant cell composition. These are listed below but do not appear to be damaging to the present project. We have appended the abstracts for these citations.

Promoting plant seed prodn. and growth - by over-expression of pyruvate phosphate di:kinase gene in plastids, SHERIFF A, EP-761816-A2 97.03.12

Prodn. of seedless plants - by over-expression of pyruvate phosphate di:kinase gene in cytosol, SHERIFF A, DE19531678-A1 97.03.06

Protein kinase gene specific in rice seeds - are used for increased prodn. of protein kinase, protein and starch, MITSUI GYOSAI SHOKUBUTSU BIO KENKYUSHO, JP06153963-A 94.06.03

accordingly there does not appear to be any issued or published patents that would govern the use f protein kinases in plants in general or the use of protein kinases to specifically alter the production of phytate in plant seeds.

DISCUSSION AND CONCLUSIONS

The current project involves two strategies for the improvement of the quality of canola seed that have substantial commercial utility. The first project is directed to the reduction of lignin in the seed coat by the use of antisense to the enzyme phenylalanine ammonia lyase. Although evidence presented by the applicants and present in the literature suggests that this approach may be successful the project faces a significant number of intellectual property barriers.

In order to commercialize products of this research in the North American and European market place licenses will be required to:

1) Use recombinant DNA constructs

2) Produce transformed *Brassica* plants

3) Use an *Agrobacterial* or equivalent transformation system

4) Use common vector components such as selectable markers and promoters

5) Use antisense RNA (or alternative methods)

In addition it is probable that a patent application from Zeneca currently under review will issue with claims that dominates the use of antisense to the PAL enzyme to reduce lignin concentration in plants. If this were to occur then an additional license would be required.

It is anticipated that licenses to the above technologies could be obtained by the applicants and that some of them may have already been secured in the course of other business activities. The production of transformed *Brassica* plants currently requires a license from Calgene that would likely cost in the range of 1 million dollars. The use of antisense RNA would probably command a similar price whereas the use of the binary *Agrobacterial* transformation system can be obtained from Mogen for approximately $450,000.00. Rights to use promoters and common vector components are anticipated to be less expensive. As several of the technologies are controlled by Calgene it may be possible to arrange a "package deal".

Opportunities to obtain patent protection for the aspects of the current project directed to lignin reduction appear modest. Published patents and patent applications and published literature comprise a significant body of dominating patents and prior art. However, none of the published documents recite the specific application of antisense to PAL for reduction of lignin in seed coats to improve the quality of seed meal. In this regard a patent with a narrow focus and claims restricted to these applications may have some possibility of success. However, as stated above, this patent would be dominated by

those already filed and even if a narrow scope patent was obtained, a license for the enabling technology would be required.

The second part of the current proposal relates to the reduction of phytate levels in seeds through intervention in phosphate metabolism. The applicants did not supply sufficient information to fully appreciate the intended experimental approach. Although it was mentioned that a mutated regulatory enzyme will be used to alter the production of phytate, the actual logic behind such an approach or the experimental evidence that supports this approach was not provided. The applicants have a long history of study in the field of plant phosphate metabolism and is assumed that the proposed approach is based on a detailed understanding of phosphate metabolism in developing seeds.

However, without more detailed information it is not possible to comment on specific intellectual property barriers to the use of mutated kinases. Dependent upon the methods used to actually mutate the enzyme, certain licenses may be required. For example, **US 5,279,952** recites a method for using PCR to construct chimeric DNA molecules (abstract and claims appended) and hence may govern the final product by virtue of its use in constructing the mutated enzyme. Similarly, selection or testing of mutated enzymes may be governed by other "methods" patents. We assume that these can be avoided, but the actual methods that will be employed are not detailed.

However the requirements described above for production of transgenic *Brassica* plants would also apply to this portion of the proposal. In principle the development of a novel method of reducing phytate based on the use of enzymes discovered by the applicants should present a good opportunity to obtain patent protection for the method. The proposed method appears to be outside of the scope of the known pending applications on plant kinases.

The potential value of low-lignin, low phytate canola can not be underestimated. Canola meal is discounted relative to soy because of these and other anti-nutritional factors. If a truly significant reduction in these two components can be made, a valuable product will result. However, it appears that commercialization of low lignin canola will require a number of licenses. The development of low phytate canola may or may not be governed by a similar number of licenses, dependent on the actual methods employed. Until these methods are defined and the actual genetic constructs that will be employed detailed, it is very difficult to provide any meaningful intellectual property assessment. The art related to the use of modified kinases in plant seeds is very new, and hence there is little in the way of relevant intellectual property. The actual methods described in the proposal give only a limited indication of the scientific rationale and results to date that are the basis for the strategy. Accordingly the intellectual property situation for this portion of the proposed project can only be defined when the actual strategy is enabled to agreater degree.